Antigen-unspecific B Cells and Lymphoid Dendritic Cells Both Show Extensive Surface Expression of Processed Antigen–Major Histocompatibility Complex Class II Complexes after Soluble Protein Exposure In Vivo or In Vitro

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Summary

Intravenous (i.v.) injection of high amounts of soluble proteins often results in the induction of antigen-specific tolerance or deviation to helper rather than inflammatory T cell immunity. It has been proposed that this outcome may be due to antigen presentation to T cells by a large cohort of poorly costimulatory or IL-12–deficient resting B cells lacking specific immunoglobulin receptors for the protein. However, previous studies using T cell activation in vitro to assess antigen display have failed to support this idea, showing evidence of specific peptide–major histocompatibility complex (MHC) class II ligand only on purified dendritic cells (DC) or antigen-specific B cells isolated from protein injected mice. Here we reexamine this question using a recently derived monoclonal antibody specific for the T cell receptor (TCR) ligand formed by the association of the 46-61 determinant of hen egg lysozyme (HEL) and the mouse MHC class II molecule I-A^k. In striking contrast to conclusions drawn from indirect T cell activation studies, this direct method of TCR ligand analysis shows that i.v. administration of HEL protein results in nearly all B cells in lymphoid tissues having substantial levels of HEL 46-61–A^k complexes on their surface. DC readily isolated from spleen also display this TCR ligand on their surface. Although the absolute number of displayed ligands is greater on such DC, the relative specific ligand expression compared to total MHC class II levels is similar or greater on B cells. These results demonstrate that in the absence of activating stimuli, both lymphoid DC and antigen-unspecific B cells present to a similar extent class II–associated peptides derived from soluble proteins in extracellular fluid. The numerical advantage of the TCR ligand–bearing B cells may permit them to interact first or more often with naive antigen-specific T cells, contributing to the induction of high-dose T cell tolerance or immune deviation.

A detailed picture now exists of the biochemical and cell biological basis of T cell antigen recognition and of the function and structure of MHC class I and class II molecules (for review see reference 1). With the extensive description of antigen processing, presentation, and recognition at the individual cell and molecular level in vitro, attention is returning to more classical immunological issues, especially the cell interactions that are involved in the evolution of T cell–dependent immune responses in vivo. Despite a large body of literature on the anatomy of lymphoid organs, leukocyte migration, and the histologic changes that accompany antigen introduction, it is only recently with the development of methods for tracking individual T and B cells of known antigen specificity that a clear view of the cellular interactions that occur after immunization has begun to emerge (2, 3).

A missing component in this emerging picture is the APC. It is clear that distinct forms of antigen and/or different routes of antigen administration to experimental animals can determine whether unresponsiveness or immunity results, as well as the quality of such an immune response. This is presumed to occur, at least in part, by favoring antigen presentation by one or another APC type (4–6). However, the study of antigen trafficking and of the role of different APCs in presenting antigen in vivo after administration by different routes has been hampered by the lack of suitable probes able to directly detect processed antigen in situ. This has made it difficult to relate APC–T cell interactions to the functional consequence of antigen exposure. As an

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indirect measure of ligand display, most studies of this issue to date have involved the isolation of candidate APCs from various tissues after antigen administration, followed by in vitro testing of the ability of these cells to activate T cells of the appropriate specificity (7–12). The major problem with this approach is that activation of a T cell is determined by factors other than just the number of available TCR ligands on the APC, such as the number and type of adhesion molecules expressed by the interacting cells or the presence of costimulatory molecules on the APC. All these factors may explain why discrepancies exist in the literature regarding the ability of various APCs to present antigens administered by the same route (7, 10).

To provide a means of directly assessing both the amount and distribution of specific peptide–MHC molecule ligands on various cells after in vivo antigen administration, we have developed a number of mAb with selective reactivity to defined peptide–MHC class I or class II combinations (Zhong, G., C. Reis e Sousa, and R.N. Germain, manuscript submitted for publication; 13). Despite some background on endogenous peptide–MHC molecule complexes, these new reagents are very effective in detecting the desired ligands on cell surfaces or within cells. We have described elsewhere the characteristics of mAb to complexes of the 46-61 and 116-129 segments of the model protein antigen hen egg lysozyme (HEL)[1] with the mouse class II A^k molecule, and the ability of these mAb to react with surface, intracellular, and detergent solubilized forms of these complexes (Zhong, G., C. Reis e Sousa, and R.N. Germain, manuscript submitted for publication).

Here we report on the use of these new reagents in flow cytometric and immunohistologic studies of antigen presentation after i.v. injection of soluble antigen without adjuvant. This route of administration of soluble proteins has been of particular interest because it allows almost immediate access of antigen to B cells, dendritic cells (DC), and macrophages throughout the body. This is thought to mimic the presentation of self-serum antigens or microbial products accessing the vascular space or extracellular fluid, and systemic antigen administration by this and other routes has been associated with the induction of either profound immunological tolerance or immune deviation to a noninflammatory response (14–18). A major outstanding question is whether these outcomes reflect the selective presentation of processed antigen by a subset of APCs, perhaps resting B cells lacking specific surface receptors for the antigen and unable to provide cosignals or cytokines necessary for response or Th1 development of antigen-specific $CD4^+$ T cells. The data available concerning this issue are contradictory, but none support the hypothesis that such B cell presentation is the dominant mode of antigen presentation in these circumstances (7, 10). In contrast to these previous results, we now demonstrate using direct measurement of TCR ligand by mAb staining that early after antigen administration, I-A^k–HEL 46-61 complexes can be detected on virtually all B cells in lymphoid tissues, independently of their surface immunoglobulin receptor specificity. Furthermore, after i.v. antigen administration, B cells and readily extracted DC in the low density fraction of spleen (LOD) display similar levels of processed antigen in association with MHC class II on their plasma membrane relative to total class II levels. Because of their numerical advantage, presentation by the large number of TCR ligand–bearing B cells may contribute to the unresponsiveness or Th2 immune deviation normally associated with the introduction of antigen into the bloodstream.

Materials and Methods

Medium. RPMI-1640 medium (Biofluids, Inc., Rockville, MD) was supplemented with 2 mM glutamine, 10 mM nonessential amino acids, 10 mM Hepes, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS (all from Biofluids, Inc.), and 10 μM 2-ME.

Mice. CBA/J and B10.BR mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used between 6 and 24 wk of age. Invariant chain–deficient mice (19, 20) backcrossed to B10.BR were a gift from Dr. E.K. Bikoff (Harvard University, Cambridge, MA).

Antigens and Immunization. HEL peptide 46-61 (NTDGSTDYGILQINSR) was synthesized and purified by the Peptide Synthesis Facility of the National Institute of Allergy and Infectious Diseases (NIH, Bethesda, MD). BSA and HEL proteins were purchased from Sigma Chemical Co. (St. Louis, MO). Mice were injected i.v. through the tail vein with HEL or BSA dissolved in PBS.

Cells. Splenocytes were prepared by dissociating spleens into a cell suspension. LOD were prepared as described (21). The low density fraction contained primarily B cells, macrophages, and DC. The latter represented ~10–15% of LOD as assessed by staining with anti-CD11c (22). B cells were depleted from LOD using magnetic beads coated with sheep anti–mouse IgG (DYNAL Inc., Great Neck, NY). DC depletion was carried out with magnetic beads coated with sheep anti–rat IgG (DYNAL Inc.), which were preincubated with 33D1 (23), NLDC-145 (24), and N418 (22) mAbs.

T Cell Hybridoma Assay. 3A9.1 is a T cell hybridoma specific for HEL 46-61–A^k (25). DC-depleted or B cell–depleted LOD were titrated in graded numbers in flat 96-well plates to which 5×10^4 3A9.1 cells were added. After overnight incubation, IL-2 secreted into the supernatant was measured by ELISA (26). Data are expressed as absorbance values obtained at 405 nm and are within the linear range of the assay.

Flow Cytometry. Whole spleen cells or LOD were stained after isolation or after overnight incubation in medium at 37°C, in the presence or absence of the indicated concentration of HEL. Cells were stained using C4H3, a rat IgG2b mAb specific for I-A^k loaded with the 46-61 peptide of HEL (Zhong, G., C. Reis e Sousa, and R.N. Germain, manuscript submitted for publication). Staining of B cells was carried out by staining first with C4H3, followed by FITC- or biotin-conjugated mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). This was followed by PE-streptavidin (Caltag Laboratories, San Francisco, CA; used in the case of the biotin-conjugated second-

[1]*Abbreviations used in this paper:* DC, dendritic cells; HEL, hen egg lysozyme; HRP, horseradish peroxidase; LOD, low density spleen; WT, wild-type.

ary antibody) and PE- or FITC-conjugated RA3-6B2 (anti-B220; PharMingen, San Diego, CA), added in a solution containing 25 μg/ml of rat IgG to block any free binding sites on the secondary antibody. Staining of DC was carried out by staining first with C4H3, followed by FITC-mouse $F(ab')_2$ anti–rat IgG; N418 supernatant (ATCC HB-224; reference 22) containing 25 μg/ml rat IgG was then added, followed by biotin-conjugated goat $F(ab')_2$ anti–hamster IgG (Jackson ImmunoResearch Laboratories) and PE- or TriColor-streptavidin (Caltag Laboratories). All staining included irrelevant, isotype-matched antibodies as negative controls and as controls for cross-reactivity. Antibody incubations ranged between 20 and 40 min on ice; washes and reagent dilutions were in cold PBS containing 1% FCS. Samples were analyzed using a FACScan™ cytometer and CellQuest™ software (Becton–Dickinson, Mountain View, CA), using propidium iodide and/or scatter gating to exclude dead cells.

Immunohistochemistry. Spleen fragments were frozen in embedding medium (Cryoform; International Equipment Co., Needham, MA). 6-μm frozen sections were cut, air-dried, fixed in acetone, and rehydrated in Tris-buffered saline containing 0.05% Tween 20. After blocking endogenous peroxidase in 0.3% H_2O_2, sections were incubated with C4H3 supernatant or an isotype-matched control, followed by horseradish peroxidase (HRP)–conjugated mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories). HRP localization was revealed using a metal-enhanced diaminobenzidine substrate (Pierce Chemical Co., Rockford, IL). For double labeling, sections were stained as above except that, before developing HRP, sections were further stained with either FITC-H57-597 (anti–TCR-β; PharMingen) or FITC-RA3-6B2 (anti-B220; PharMingen), added in a solution containing 25 μg/ml of rat IgG; this was followed by alkaline phosphatase–conjugated rabbit antifluorescein (BIODESIGN International, Kennebunk, ME). Alkaline phosphatase was developed first, using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.), and HRP was developed last, using the same substrate as for single staining. Sections were dried and mounted in Permount (Fisher Scientific Co., Fairlawn, NJ). Stained sections were photographed on an Axiophot compound microscope (Carl Zeiss Inc., Thornwood, NY) using Kodachrome 25 film (Eastman Kodak Co., Rochester, NY).

Results

Processing and Presentation of HEL by B Cells and DC after Antigen Exposure In Vitro. In cells that have not been exposed to HEL, the C4H3 mAb stains a subset of I-A^k molecules loaded with certain self peptides (background staining), but the staining is markedly increased on HEL-pulsed cells, in proportion to the number of A^k complexes produced containing the 46-61 peptide or overlapping segments of the HEL protein (referred to throughout as HEL 46-61–A^k based on the known immunodominance of this particular segment of the HEL protein; reference 27) (Zhong, G., C. Reis e Sousa, and R.N. Germain, manuscript submitted for publication). The relative surface expression of HEL 46-61–A^k complexes by DC and B cells was assessed by using C4H3 to stain whole spleen or LOD that had been incubated overnight in vitro in the presence or absence of a subsaturating concentration of soluble HEL. As shown in Fig. 1, DC cultured in medium alone have more HEL-independent staining than B cells, due largely to the fact that they express higher absolute numbers of I-A^k molecules on the plasma membrane (Reis e Sousa, C., unpublished observations). However, most DC and B cells display substantial levels of HEL 46-61–A^k complexes on their surface after pulsing with intact antigen, reflected in increased staining with C4H3 (Fig. 1). Interestingly, the relative magnitude of this increase as a multiple of the initial background staining does not differ significantly between whole spleen (small, resting) B cells and low density (larger, activated) B cells and is not very different between B cells and DC (Fig. 1). Although careful analysis of mean fluorescence ratios reveals that the proportional increase in B cell staining is actually slightly greater than that seen with DC, the absolute level of specific ligand is somewhat higher on DC, in agreement with their higher overall level of MHC class II expression.

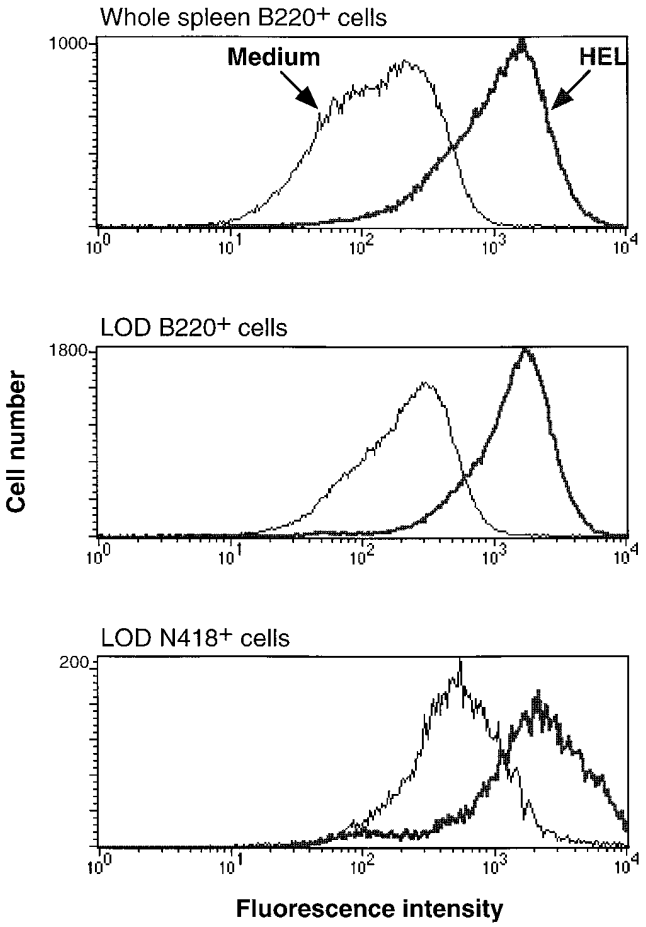

Figure 1. B cells and DC present processed HEL in vitro with similar efficiency. Collagenase-treated spleens from five CBA/J mice were dissociated into a cell suspension and part of this suspension was fractionated over dense BSA columns to obtain LOD. Whole splenocytes (*top panel*) and LOD (*middle and bottom panels*) were incubated overnight in medium alone or containing a nonsaturating concentration of HEL (1 mg/ml). Cells were recovered and triple stained with C4H3, B220 and N418. Profiles represent the C4H3 fluorescence of gated $B220^+$ $N418^-$ cells (*top and middle panels*) or gated $N418^+$ $B220^-$ cells (*bottom panel*); profiles from cells incubated in the absence of HEL are represented by thin lines and those from HEL-pulsed cells by thick lines.

Processing and Presentation of HEL by B Cells In Vivo After i.v. Antigen Administration. Although B cells are known to take up and present soluble proteins in vitro independently of their sIg specificity (for review see reference 28 and Fig. 1), several investigators have failed to find evidence for this phenomenon in vivo under noninflammatory conditions (7, 10). To reexamine this issue and assess the capacity of C4H3 to reveal TCR ligand–bearing cells in situ, we injected CBA/J mice i.v. with HEL or a control protein, BSA, and removed the spleens 4 h later. Part of the spleen was frozen, sectioned, and stained with C4H3. As shown in Fig. 2 *A*, C4H3 fails to stain spleen sections from BSA-injected mice, except for a few scattered profiles in the T cell area, corresponding to a subpopulation of interdigitating DC that appear to have elevated numbers of self-antigen loaded class II molecules reactive with these reagents (Reis e Sousa, C., G. Zhong, and R.N. Germain, manuscript in preparation). In contrast, dramatic staining of a large fraction of the white pulp is seen in sections from HEL-injected mice (Fig. 2 *B*). By double labeling, C4H3 staining is found to be adjacent to staining of the T cell areas of spleen (Fig. 2, *C* and *D*) and to overlap entirely with

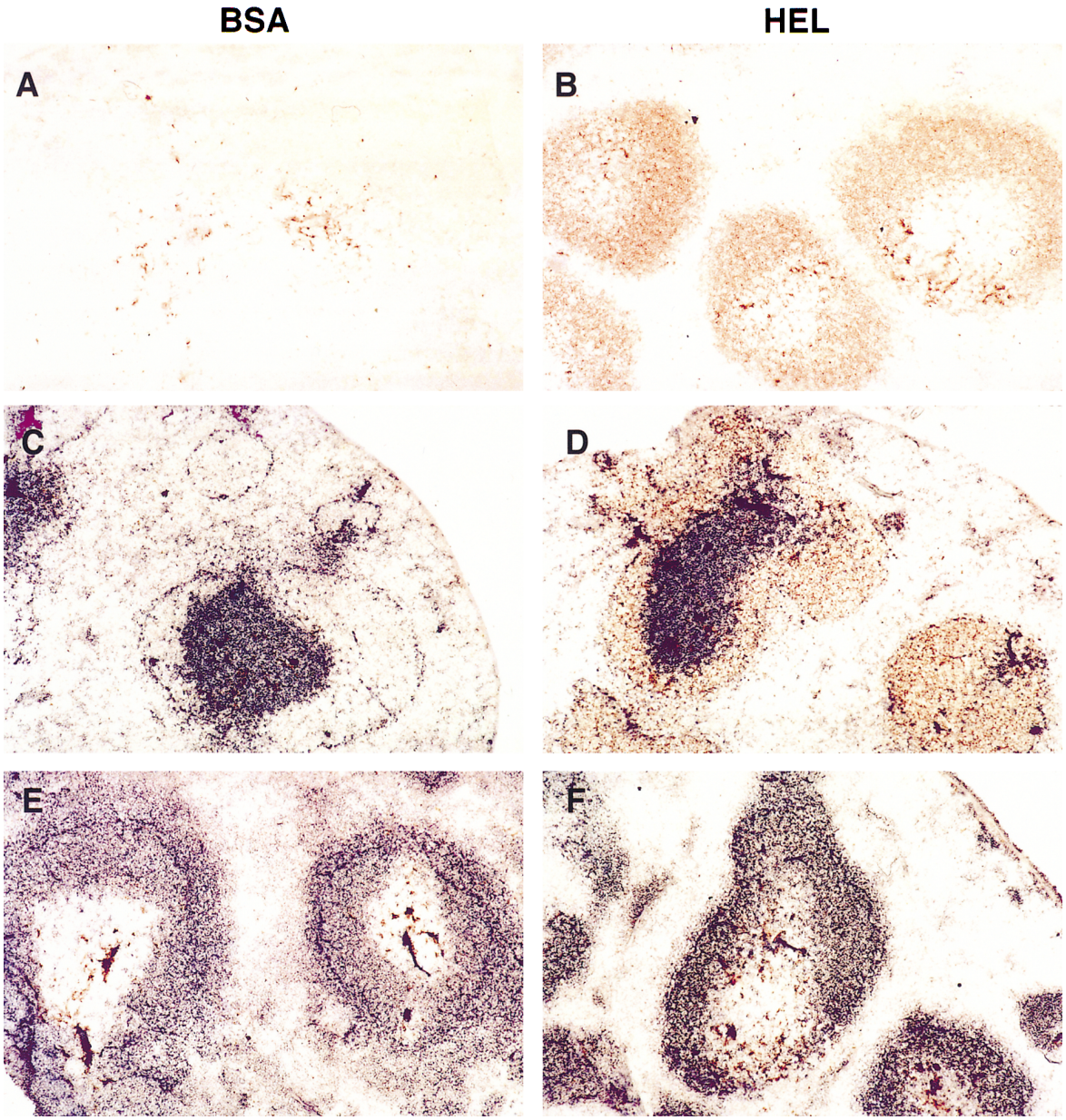

Figure 2. Visualization of APC bearing processed HEL in situ. (*A–F*) Frozen sections of spleen from CBA/J mice injected i.v. 4 h previously with 8 mg BSA (*A*, *C*, and *E*) or HEL (*B*, *D*, and *F*) were stained with C4H3 (*brown/red color*). Sections were single stained (*A* and *B*) or double stained (*blue*) with an anti-TCR-β antibody to define T cell areas (*C* and *D*) or an anti-B220 antibody to define B cell areas (*E* and *F*). Note staining of B cells with C4H3 in HEL-injected but not in control animal. Double stained B cells appear blue–black (*F*).

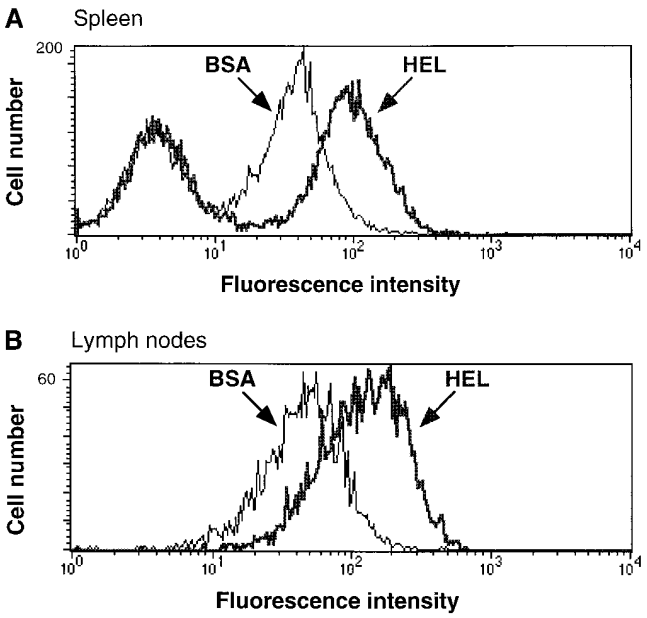

Figure 3. B cells capture and present HEL injected i.v. independently of receptor specificity. (*A*) B cell staining in the spleen is due to presentation of surface HEL 46-61–A^k complexes. Part of the spleen from each animal used in the experiment summarized in Fig. 2 was dissociated into a cell suspension and stained with C4H3. (*B*) HEL presentation by virtually all B cells is also seen in lymph nodes. Superficial inguinal lymph nodes from B10.BR mice injected with 8 mg BSA or HEL were dissociated into a cell suspension and double stained with C4H3 and B220. Data for splenic B cells from the same experiment are summarized in Table 1. Profiles in *A* represent total spleen cell staining and profiles in *B* represent the C4H3 fluorescence of gated $B220^+$ cells; BSA-injected mouse, *thin line*; HEL-injected mouse, *thick line*.

staining of the B cell areas (Fig. 2, *E* and *F*). This dramatic result suggests that the bulk of the B cells in the spleen of these mice, irrespective of surface Ig antigen specificity, display high levels of HEL 46-61–A^k complexes after i.v. antigen injection.

To confirm this result and to determine if these complexes were present at the cell surface, the remainder of the spleen from the same mice was analyzed by flow cytometry after staining with the same antibody. A distinct subpopulation of spleen cells from HEL-injected mice shows an increase in staining with C4H3 over the background seen on the same cell subpopulation from control mice (Fig. 3 *A*), consistent with the interpretation of the immunohistochemical data that essentially all B cells present processed HEL at the cell surface. A 1.5–20-fold increase in B cell staining with C4H3 (mean fluorescence) after i.v. injection of doses of 2–8 mg HEL was seen reproducibly in more than seven independent experiments and could be detected as early as 1–2 h after injection (data not shown). The same increase in staining could be seen in B cells isolated from other tissues, including lymph nodes (Fig. 3 *B*), blood, bone marrow, and Peyer's patches (data not shown). Thus, these results demonstrate that most antigen-unspecific B cells present peptides derived from protein antigens injected i.v.

To investigate whether B cell display of HEL 46-61–A^k complexes under these experimental conditions was due to

Table 1. *Processing Intact HEL by B Cells after i.v. Injection Is Invariant Chain–dependent*

	Antigen		
Mouse	BSA	HEL	HEL 46-61
WT	100	290	101
IiKO	100	114	131

WT B10.BR or invariant chain knockout (IiKO) mice were injected i.v. with 8 mg of BSA or HEL protein (equivalent to 0.56 μmol HEL), or with 0.5 μmol of HEL 46-61 peptide. After 4 h, spleens were removed and splenocyte suspensions were double stained with C4H3 and B220 and analyzed by flow cytometry. The mean C4H3 fluorescence of gated $B220^+$ cells is expressed as a percentage of the BSA-injected controls to account for the differences in MHC class II expression between IiKO and WT animals. The absolute mean fluorescence values for the BSA-injected controls, after subtracting the background obtained with rat IgG alone, were: WT, 50.24, IiKO, 4.67.

active intracellular processing and not to the activity of serum or cell surface proteases, followed by binding of the resultant peptides to I-A^k on the B cell surface, we examined whether staining was dependent on the presence of invariant chain. Wild-type (WT) B10.BR or invariant chain–deficient mice (19, 20) were injected i.v. with HEL or BSA and analyzed as before. WT B10.BR mice injected with HEL show an increase in C4H3 staining of their splenic B cells compared to control mice injected with BSA (Table 1), confirming the results obtained with CBA/J mice. In contrast, virtually no increase in staining is seen after HEL injection into the mutant mice (Table 1). Because B cells in invariant chain–deficient mice have lower levels of MHC class II surface expression (19), it remained possible that the lack of increase in staining in the spleens of such mice reflected the lower abundance of surface sites for binding of peptides generated extracellularly. This is not the case, however, because I-A^k on these mutant B cells is more easily loaded with exogenous peptides injected i.v. than I-A^k on WT B cells (Table 1), as previously reported using in vitro assays (19). The fact that HEL-dependent staining of B cells is not seen in invariant chain–deficient mice despite this biasing of the experiment towards detection of extracellular processed antigen argues that nearly all B cells, irrespective of surface Ig specificity, take up and actively process HEL intracellularly upon i.v. administration and subsequently present the processed antigen at the cell surface in association with A^k.

DC also Present HEL after i.v. Administration. The ability of splenic DC to process antigens after i.v. immunization remains controversial (7, 10). To compare directly the presentation of HEL by DC and B cells, we used flow cytometric analysis of cells stained with C4H3. Spleens were removed from groups of mice that had been injected i.v. 4 h before with either BSA or a subsaturating amount of HEL and whole or low density spleen cells were stained with C4H3 followed by double staining with either anti-DC or anti–B

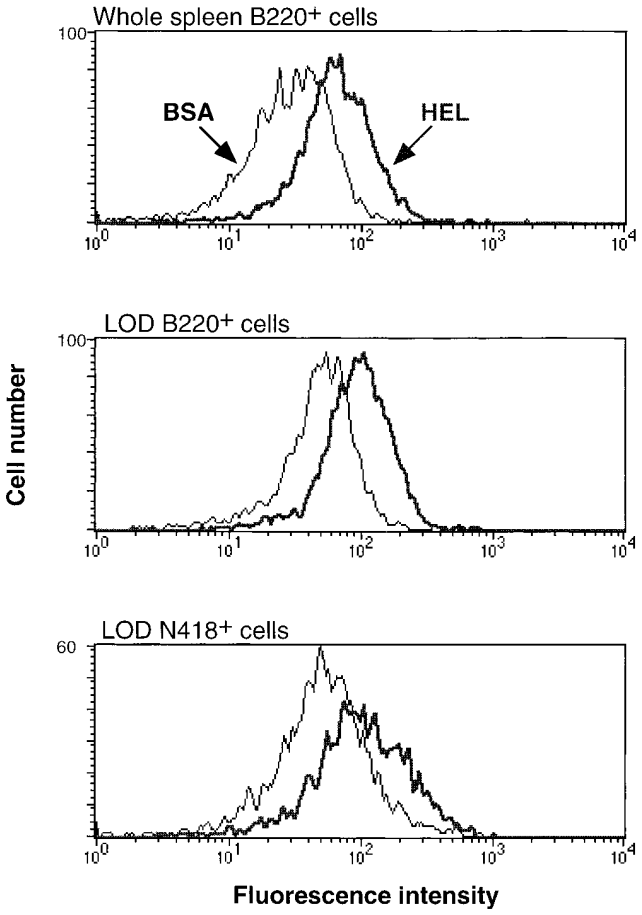

Figure 4. Both B cells and DC present processed HEL after i.v. injection. Groups of five CBA/J mice were injected i.v. with 8 mg of BSA or HEL in 100 μl PBS. Spleens were removed 4 h later and were dissociated into a cell suspension using collagenase. Part of this suspension was fractionated over dense BSA columns to obtain LOD. Whole splenocytes (*top panel*) and LOD (*middle and bottom panels*) were double stained with C4H3 and B220 or C4H3 and N418. Profiles represent the C4H3 fluorescence of gated $B220^+$ cells (*top and middle panels*) or gated $N418^+$ cells (*bottom panel*); profiles from BSA-injected mice are represented by thin lines and those from HEL-injected mice by thick lines.

cell antibodies. Overlapping the profiles from HEL- and BSA-injected mice demonstrates an HEL-dependent increase in C4H3 staining for splenic B cells (Fig. 4, *top panel*), as seen before (Fig. 3 and Table 1). As with antigen exposure in vitro (Fig. 1), the increase is similar for whole spleen and low density B cells and for DC when considered as a fraction of total class II expression (Fig. 4). In three out of three experiments, whole spleen B cells were consistently slightly better than either low density B cells or DC by this criterion, whereas the differences between the latter two APCs were small and inconsistent. We conclude that both DC and B cells in the spleen are capable of processing and presenting peptides derived from soluble proteins administered i.v. and that under nonactivating conditions of antigen exposure, do so with similar relative efficiency. As with the in vitro experiments, however, DC again have a higher absolute level of specific ligand expression.

To confirm that the display of HEL 46-61–A^k complexes by DC and B cells detected with C4H3 represents ligands capable of stimulating T cells, spleen DC and B cells from mice injected with HEL were compared for their ability to stimulate 3A9.1, a T cell hybridoma whose activation is relatively independent of costimulation (25). As shown in Fig. 5 *A*, DC-depleted LOD contain 10-fold fewer $N418^+$ cells than B cell–depleted LOD (4 vs. 43%) and, conversely, B cell–depleted LOD contain 40-fold fewer $B220^+$ cells than DC-depleted LOD (1 vs. 41%). Both APC populations isolated from HEL-injected but not from BSA-injected mice are able to activate 3A9.1 cells (Fig. 5 *B*; compare *left* and *right*), as expected from the ability of both to be stained to nearly comparable levels with C4H3 (not shown; see Fig. 4). Furthermore, both APC types stimulate 3A9.1 with a similar dose response curve (Fig. 5 *B*). Since APCs were titrated into the assay as a function of their original number in the starting LOD fraction, this result suggests that DC are about fivefold better on a per cell basis than B cells at stimulating 3A9.1 cells, because DC represent about one-fifth the number of B cells in LOD preparations (~10 vs. ~50%).

Discussion

Tracking antigen-specific cellular interactions in vivo is essential to a thorough understanding of the adaptive immune system. This has proven particularly challenging because lymphocytes specific for a given antigen are both extremely rare and difficult to identify. This problem has been resolved, in part, by following responses in which a predominant use of certain V region segments has been documented (29, 30). Other investigators have used adoptive transfer models in which murine T or B cells expressing transgenic receptors specific for a given antigen are transferred into naive recipients, artificially increasing the frequency of antigen-specific cells and allowing them to be tracked after antigen administration with antibodies to the clonotypic receptors, or in the case of B cells, with labeled antigen itself (31, 32). However, a remaining stumbling block in characterizing the events that occur after antigen administration has been the inability to track APCs which carry the processed, MHC molecule–associated antigen recognized by the transgenic TCR in situ. Several groups have used the localization of administered labeled antigens with respect to the distribution of MHC class II-bearing cells as a surrogate for antigen presentation to $CD4^+$ T cells (for review see reference 33). However, this approach detects endocytic accumulation of the offered antigen rather than the peptide–MHC molecule complexes themselves. Such antigen-positive cells may fail to load processed peptides effectively into class II molecules; conversely, some APCs such as DC can present antigen to T cells in experimental conditions in which antigen uptake is barely detectable (34).

Alternative approaches have involved isolating cells from various tissues after antigen administration and testing them

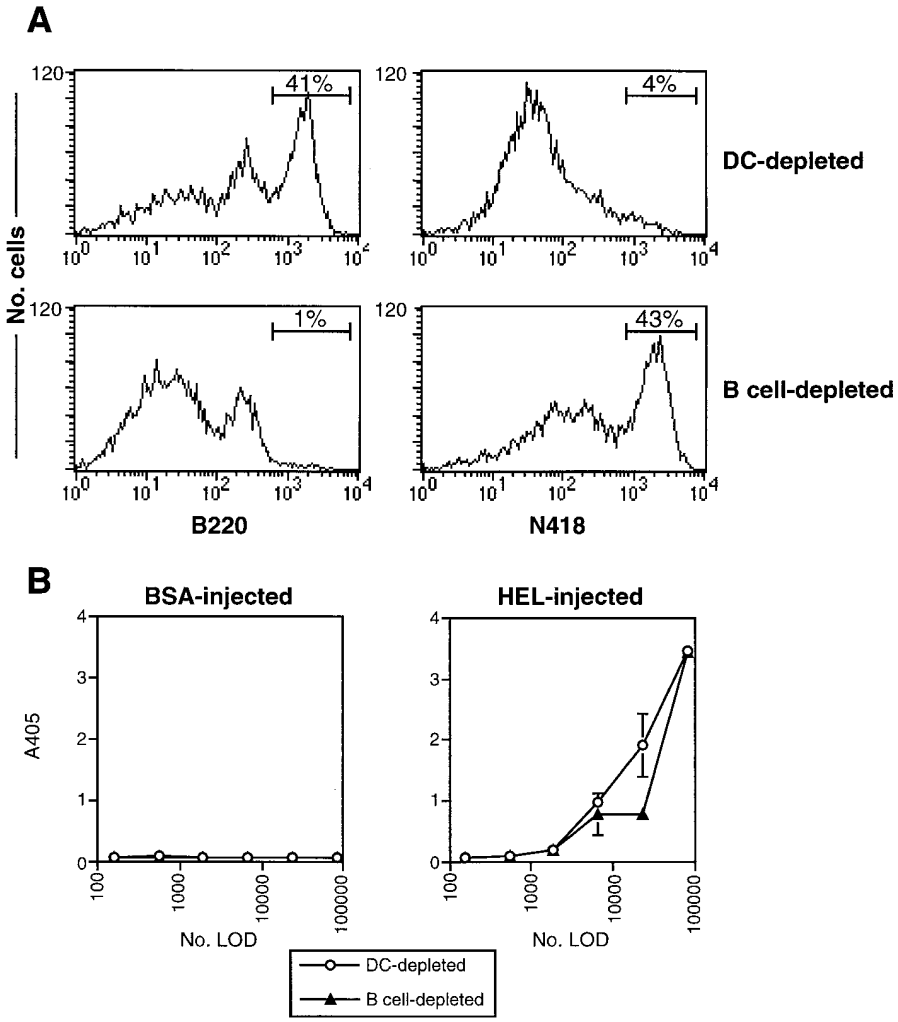

Figure 5. Presentation of i.v. injected HEL by both B cells and DC can be detected using an HEL-specific T cell hybridoma. LOD were prepared as in Fig. 4 from groups of mice injected i.v. 4 h previously with 4 mg HEL or BSA, then depleted of DC or B cells with magnetic beads as detailed in the Materials and Methods section. (*A*) Depletion as assessed by flow cytometry after staining with B220 or N418 as indicated; the percentages of $B220^+$ or $N418^+$ cells are indicated above each profile. Data shown are for depletion of LOD from mice injected with HEL; similar results were obtained with LOD from BSA-injected mice (not shown). (*B*) Both DC-depleted and B cell–depleted LOD can present HEL 46-61–A^k complexes to the 3A9.1 T cell hybridoma. Stimulation of 3A9.1 was assessed after overnight incubation with graded numbers of APC from BSA-injected (*left*) or HEL-injected (*right*) mice. Numbers of APCs titrated reflect the numbers of cells in the starting LOD population and were not adjusted after depletion. Stimulation of 3A9.1 cells is represented by the absorbance values obtained in the IL-2 ELISA. Data are representative of three independent experiments.

for the ability to present the antigen to T cells in vitro (7–12). These are laborious experiments which only indirectly assess the actual ligand display of the cells, as such T cell assays depend on a host of other APC–T cell interactions to translate TCR engagement into functional responses which can be measured. Furthermore, such assays cannot differentiate between increased levels of antigenic complexes per cell versus an increased frequency of subpopulations of cells bearing antigenic complexes.

In this study, we have addressed a question explored previously by several groups using T cell assays, namely what cell population(s) bear(s) foreign peptide–MHC class II complexes after systemic administration of soluble protein antigen in the absence of adjuvant. This is a model for induction of "high-zone" tolerance (35) or immune deviation (15–18) that has been widely studied. Some investigators have argued that this route favors rapid and extensive presentation of antigen by resting B cells lacking costimulatory properties or cytokine production that overwhelms effective presentation by activating DC, resulting in clonal anergy, deletion, or deviation towards the Th2 phenotype (4–6). However, using T cell assays to detect cells bearing processed antigen, two groups failed to find evidence for antigen presentation by polyclonal B cells after injection of myoglobin or conalbumin (7), or pigeon cytochrome *c* or a cytochrome *c*-HEL conjugate (10). In both studies, the absence of processed antigen on B cells was inferred from the inability of these cells to induce proliferation of naive transgenic T cells (10), normal T cell clones, or primed lymph node T cells (7).

However, T cell proliferation is extremely dependent on costimulation for IL-2 production (36). Thus, T cell proliferation assays are naturally biased to detect antigen on APC such as DC which express CD28 ligand(s) upon in vitro culture, rather than on APCs such as resting B cells which are deficient in providing signal 2 (37, 38). The results of these T cell assays have been interpreted in one case as unequivocally indicating an absence of significant levels of processed antigen on antigen-unspecific B cells under these conditions (10), whereas in another, the lack of T cell stimulation was ascribed to either the absence of such TCR ligand (signal 1) on the surface of the B cells, or the inability of these cells, in contrast to DC from the same mice, to provide other signals to activate T cells (signal 2) (7). Very

recently, in studies which involve analysis of cells from lymph nodes draining sites of inflammation induced by adjuvants, Guéry et al. (12) have reported that i.v. injection of HEL allows presentation by B cells but not DC, using for their assay transformed T cells that are less costimulation sensitive.

In contrast to these studies, we have here analyzed antigen presentation by various cell types after in vitro and in vivo soluble antigen administration using a direct assay for specific peptide–MHC class II complexes involving staining with mAb to TCR ligands. This approach obviates the problems associated with indirect T cell assays as a means of evaluating antigen presentation. Using this approach with flow cytofluorimetry or immunohistology, we report clear evidence for extensive HEL presentation by a large fraction of B cells in spleen, lymph nodes, and other lymphoid organs in noninflammatory conditions (Figs. 2–4). Essentially all $B220^+$ B cells bear processed HEL on their surface in association with A^k as early as 1–4 h after i.v. injection of milligram quantities of HEL protein (Figs. 2, 3, and 4, and data not shown). Using invariant chain–deficient mice, we demonstrate that this polyclonal B cell presentation is due to active intracellular antigen processing (Table 1). Our results using peptide–MHC-specific antibodies are consistent with the alternative interpretation given in some earlier studies of B cell versus DC presentation after i.v. antigen exposure, which suggests that differential cosignaling, rather than differential TCR ligand display, accounts for the failure of B cells to activate the responding T cells in vitro. Consistent with that interpretation, we find evidence for polyclonal B cell presentation after i.v. immunization not only by direct staining for processed antigen but also by using B cells to stimulate a T cell hybridoma relatively insensitive to costimulation (Fig. 5).

It needs to be stressed that the high generalized level of B cell ligand display we measure here occurs only with substantial concentrations of offered HEL protein, and may not be seen at limiting antigen concentrations. Antigen-specific B cells are much more efficient at presenting low amounts of antigen in vitro than B cells not bearing surface Ig reactive with the antigen (for review see reference 28). Polyclonal B cell presentation of HEL in our current system is likely to be mimicking presentation of high-abundance serum proteins taken up through the normal pinocytic activity of B cells rather than presentation of the low concentrations of antigen available at very early or very late times during an infection, when surface Ig capture and concentration of antigen may be most important.

Our results also help resolve the controversy over whether DC in the spleen process and present intact antigens administered i.v. (7, 10). Earlier results arguing that DC were the main APC capable of stimulating T cells after systemic administration of protein antigens (7) were not reproduced in a later study by a different group (10). In contrast, the later study demonstrated that DC were the main APC isolated from peptide-injected, but not intact protein-injected, mice which were capable of stimulating T cells in vivo (10). This suggested that splenic DC could not process intact antigen, but could be easily loaded with exogenous peptides, arguing that the apparent processing of intact antigen by DC in the earlier study had been an artifact arising from the presence of peptide contaminants in the injected protein preparations (10). These results are in line with a recent report showing that, after i.v. administration of HEL, B cells in immune lymph nodes or draining sites of adjuvant administration can present that antigen whereas DC from the same lymph nodes cannot (12). In contrast, our results show that DC isolated from spleen bear substantial numbers of HEL 46-61–A^k complexes on their plasma membranes after i.v. administration (Fig. 4) in a system in which peptide-free protein solutions are used and extracellular proteolysis does not generate enough peptide to give detectable staining (Table 1 and data not shown). Moreover, flow cytometric comparison of the staining of B cells and $N418^+$ DC fails to reveal significant differences in the TCR ligand levels relative to total I-A^k levels displayed by the two cell types (Fig. 4), suggesting that splenic B cells and splenic DC have a similar ability to process and present serum proteins under the nonactivating conditions we have used.

This does not mean that all DC and B cells have the same ability to capture and present antigen. DC isolated from spleen are thought to represent a more mature form of DC, composed of recent immigrants from nonlymphoid tissues that have downregulated the ability to capture and process intact antigens (39, 40), although they have not entirely lost this functionality (34, 41). In comparison, truly immature DC, such as Langerhans cells, are much more efficient at taking up and processing antigens (41, 42) and, in fact, immature DC have been claimed to be as good as or better than antigen-specific B cells at capturing and presenting limiting concentrations of antigen (43). In most forms of antigen administration which lead to an immune response, antigen is probably taken up in nonlymphoid tissues by immature DC that migrate to the lymph nodes or spleen (39, 40), and only a limiting amount of free antigen drains to those tissues, where it may be preferentially picked up by antigen-specific B cells, rather than polyclonal B cells or lymphoid DC.

Although our results show that the relative increment over background of specific peptide–MHC class II complexes is similar for B cells and lymphoid DC after i.v. HEL administration, DC nonetheless display more total antigenic complexes on their surface by virtue of their higher total expression of MHC class II molecules. This is clear from the greater intensity of C4H3 staining of HEL-pulsed DC relative to B cells (Figs. 1 and 4) and raises the interesting question of whether T cells simply integrate the total number of antigenic complexes on the surface of an APC, irrespective of other peptide–MHC complexes that might be present, or whether the fraction of total complexes loaded with the right peptide is a determining factor in T cell activation. In the former case, DC would clearly have an advantage over B cells at providing signal 1, even if their pro-

cessing efficiency were lower. Analysis of functional antigen presentation by LOD DC versus LOD B cells showed about a fivefold advantage for DC over B cells on a per cell basis, whereas the absolute difference in specific antigen complexes was only about twofold. These data are consistent with the notion that DC have a variety of advantages beyond absolute ligand display, such as adhesion coligands and costimulatory molecules, which improve their function as APCs (39).

I.v. injections are an artificial mode of antigen introduction that may simulate the presentation of normal serum proteins and of pathogen products present in extracellular fluids. This form of antigen exposure is more often associated with induction of tolerance or deviation to Th2 responses than with inflammatory-type immunity (14–18). The observations reported here support a previously suggested mechanism by which i.v. injections can promote such immune effects (4–6). Because antigen-unspecific B cells are more abundant than DC in the spleen, if both APC present the injected antigen to a similar extent, it is likely that on the basis of numbers alone antigen-specific T cells will encounter the antigen on a B cell before seeing it on a DC, perhaps being anergized, clonally eliminated, or deviated to a Th2 differentiation pathway (5, 6). Testing of this model by directly visualizing the interactions between HEL-specific T cells and antigen-bearing APCs using mAbs specific for processed HEL is currently underway.

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